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Resistance of Spanish codling moth (*Cydia pomonella*) populations to insecticides and activity of detoxifying enzymatic systems

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Abstract

Resistance of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) to insecticides has become a major problem in many apple and pear production areas. Our aim was to determine the level of insecticide resistance in Spanish field populations. Seven field populations collected from apple, *Malus domestica* Borkhausen (Rosaceae), orchards and three laboratory susceptible strains of codling moth were studied. Damage at harvest in all the conventional orchards from which codling moth populations were collected was higher than the economic threshold. The efficacy of eight insecticides, with five modes of action, was evaluated by topical application of the diagnostic concentrations on post-diapausing larvae. The enzymatic activity of mixed-function oxidases (MFOs), glutathione transferases (GSTs), and esterases (ESTs) was evaluated for each population. The susceptibility to insecticides and the biochemical activity of the three laboratory strains and one organic orchard population were not significantly different. Field populations were less susceptible to the tested insecticides than the susceptible strains, especially for azinphos-methyl, diflubenzuron, fenoxycarb, and phosalone. The efficacy of all insecticides was significantly dependent on the activity of MFOs. Only the toxicity of the three insecticides most used in Spain when the populations were collected (azinphos-methyl, fenoxycarb, and phosalone) was also dependent on the activity of ESTs and GSTs activity. According to our results, the control failures were due to the existence of populations resistant to the main insecticides used.

Introduction

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a worldwide key pest in apple, pear, quince, and walnut orchards in temperate regions (Shel'Deshova 1967). The geographic origin of this pest is Eurasia, from where it has spread to European regions, North and South America, South Africa, Australia, and New Zealand (Franck et al., 2007), but it is not present in Japan and in some regions of China (Croft, 1982). Control strategies are based on the application of chemical insecticides, either alone or in combination with other techniques, such as mating disruption or microbial control. Several insecticide groups are available, depending on country or region. They include nerve action products (organophosphates, carbamates, pyrethroids, neonicotinoids, and spinosyns), nerve and muscle action products (avermectins and diamides), lipid synthesis action products (tetramic acid derivatives), and insect growth regulators (chitin synthesis inhibitors, juvenile hormone analogues, and ecdysone receptor agonists).

A reduction in insecticide efficacy on *C. pomonella* populations was recorded for the first time for arsenates (Hough, 1928). Since then, in almost all the main apple-growing regions worldwide, field populations of codling moth have developed resistance to insecticides such as organophosphates, carbamates, pyrethroids, neonicotinoids, avermectins, benzoylureas, juvenile hormone analogues, and ecdysone receptor agonists (Moffitt et al., 1988; Bush et al., 1993; Knight et al., 1994; Chapman & Barrett, 1997; Sauphanor et al., 1994, 1998, 1999, 2000; Bouvier et al., 2001; Reyes et al., 2007). We have little knowledge of insecticide resistance in Spanish populations of codling moth, but reduced efficacies of several insecticides have been recorded (Bosch et al., 1999; Reyes et al., 2007; Reyes & Sauphanor, 2008; Rodríguez et al., 2010).

The resistance of codling moth populations to insecticides may be due to the action of enzymes or to modifications of the insecticide molecular targets. Mixed-function oxidases (MFOs) and/or glutathione transferases (GSTs) have been demonstrated to be responsible for resistance in codling moth populations (Sauphanor et al., 1997; Bouvier et al., 2002; Reyes et al., 2007; Fuentes-Contreras et al., 2007). The role of esterases (ESTs) is less clear, as populations resistant to organophosphates may show an EST activity that is lower [USA (Bush et al., 1993), France (Bouvier et al., 1998)] or higher [Argentina (Soleño et al., 2004, 2008), Spain (Rodríguez et al., 2010)] than that of susceptible populations. The modifications of the molecular targets reported are a *kdr* mutation which gives resistance to deltamethrin by L1014F substitution in the voltage-dependent sodium channel protein (Brun-Barale et al., 2005), and a mutation by a single amino acid (F290V) replacement in acetylcholinesterase

(AChE), which is involved in the resistance to azinphos-methyl and carbaryl (Cassanelli et al., 2006). The latter mechanism has been detected only in Spanish populations (Reyes et al., 2009).

Codling moth resistance to insecticides should be detected in eggs or neonate larvae, which are the target instars of the insecticide applications. However, bioassays based on the topical application of the insecticide on post-diapausing larvae using pre-established diagnostic concentrations are an efficient tool for detecting resistance (Pasquier & Charmillot, 2003). In recent years, several studies have applied this methodology (Reyes et al., 2004, 2007; Charmillot & Pasquier, 2005; Charmillot et al., 2007; Ioriatti et al., 2007; Fuentes-Contreras et al., 2007; Rodríguez et al., 2010), because high numbers of post-diapausing larvae are easy to collect, their conditions of age and susceptibility are homogeneous (Pasquier & Charmillot, 2003), and a relatively small number of individuals per bioassay and per insecticide is needed. Moreover, a recent study showed that a bioassay on post-diapausing larvae of codling moth is suitable for routine monitoring and an efficient means of preventing the risk of resistance to insecticides and the risk of developing cross-resistance (Reyes & Sauphanor, 2008).

The aim of this study was to detect the presence in Spanish apple orchards of *C. pomonella* populations resistant to eight insecticides, with five different modes of action. For this purpose, we used topical application to perform a post-diapausing larval bioassay and measured the activity of the insecticide detoxifying enzymes.

Materials and methods

Codling moth field populations and susceptible laboratory strains

Field *C. pomonella* populations were collected from apple orchards located in the apple production areas of Lleida and Girona (NE Spain). They were collected from five conventional insecticide-treated orchards (Poal_1 (41º 40’ N; 0º 51’ E; 215 m altitude); Poal_3 (41º 40’ N; 0º 52’ E; 221 m altitude), Poal_4 (41º 41’ N; 0º 53’ E; 226 m altitude), Bellvís_2 (41º 40’ N; 0º 48’ E; 204 m altitude) and Girona (42º 10’ N; 3º 05’ E; 1 m altitude)), one mating disruption + insecticide–treated orchard (Gimenelles (41º 39’ N; 0º 23’ E; 258 m altitude)), and one organic, mating disruption + granulovirus (CpGv)–treated orchard (Boldú (41º 43’ N; 1º 00’ E; 264 m altitude)). The orchards were selected because codling moth populations were high and the percentage of injured apples during the season was high in spite of the control measures adopted. Three susceptible laboratory strains (named

S_Spain, S_France, and S_Italy) were used in this study. They were collected from abandoned orchards of Lleida (Spain 41º 41' N; 0º 38' E; 213 m altitude), Les Vignères-Avignon (France 43º 52' N; 4º 59' E; 57 m altitude), and Molinella-Bologna (Italy 44º 37' N; 11º 40' E; 5 m altitude) and reared on semi-artificial diets without any insecticide exposure for at least 12 years. S_France and S_Italy were received in the laboratory of the UdL-IRTA Centre for R+D, Spain, in 2004 and 2005, respectively, and have been reared since then on the same diet and under the same conditions as S_Spain.

Insecticides

Eight insecticides were tested using solutions of the technical materials in organic solvents (Table 1). Diflubenzuron was dissolved in tetrahydrofuran (stabilized with 0.025% BHT; J.T. Baker, Barcelona, Spain), whereas all the other insecticides were dissolved in acetone (for organic residue analysis, 99.4% purity; J.T. Baker).

Insect management

More than 250 diapausing larvae per orchard were collected in late summer–early autumn 2005 using corrugated cardboard bands placed around the tree trunks. Post-diapausing larvae from the F1 generation reared in the laboratory were used in the experiments. To obtain them, all the collected diapausing larvae were placed into new corrugated cardboard bands and kept in plastic boxes for 2-3 months at 6 ± 1 ºC and a photoperiod of L12:D12, which are conditions that break the diapause. After this period, the post-diapausing larvae were maintained at 22 ± 3 ºC and L16:D8 until the adults emerged. All the adults that emerged (more than 200 per population) were then confined in rearing cages in order to obtain eggs. The eggs were removed three times a week and transferred to plastic boxes with moist filter paper. The neonate larvae were individually put in plastic boxes (5 cm diameter, 3 cm high) with a dehydrated, apple-based, semi-artificial diet (Pons et al., 1994), and were maintained at 25 ± 3 ºC and L12:D12 for 40 days to obtain the F1 diapausing larvae. The diapausing larvae were kept for at least 3 months at 6 ± 1 ºC and L12:D12 in order to satisfy the chilling requirements for diapause breaking. The post-diapausing larvae were placed at 22 ± 3 ºC and L16:D8 for 24 h, before being used for the bioassays or for the analysis of the enzymatic activity. The same procedure was applied to obtain post-diapausing larvae from the susceptible strains.

145 **Insecticide efficacy**

146 The efficacy of each insecticide was tested at the diagnostic concentration shown in Table 1.
147 The tested diagnostic concentrations have been reported to cause 72–99.9% mortality in a
148 susceptible Swiss population (S_Switzerland) (Pasquier & Charmillot, 2003; Charmillot et al.,
149 2007). A 1-µl drop of the insecticidal solution – or of the organic solvent in the case of the
150 controls – was applied with a Multipette Plus (Eppendorf) in the dorsal median region of the
151 larva (Sauphanor et al., 2000). Immediately after the treatment, 10 larvae were transferred
152 inside 20 × 20 mm pieces of corrugated cardboard placed within 90-mm-diameter Petri
153 dishes, and were kept at 22 ± 3 ºC and L16:D8. The Petri dishes were checked daily until 15
154 days had elapsed since the last adult emergence, and the mortality was then recorded. Four
155 replicates of 10 larvae each were carried out per population and insecticide. Depending on the
156 sample size, 3-8 insecticides were tested per field population.
157
158 **Enzymatic activity**

159 Twenty post-diapausing larvae were used per population and enzymatic complex. Mixed-
160 function oxidase (MFO) activity was measured by fluorescence, and glutathione transferase
161 (GST) and esterase (EST) activity was measured by absorbance (Bouvier et al., 2002), using a
162 VICTOR^3 Multilabel Plate Counter (PerkinElmer Life and Analytical Sciences, Madrid,
163 Spain).

164 The MFO activity was analyzed with an in vivo protocol. Post-diapausing larvae were
165 individually placed on ice in a 6 g l^{-1} sodium chloride solution, and were cut, using micro-
166 scissors, into four fragments in order to prevent over-expression of the enzymatic activity per
167 well. Each fragment was deposited individually in a well and used as the enzymatic source.
168 The results of the four fragments were added to obtain the enzymatic activity per individual.
169 The GST and EST activities were also analyzed with an in vivo protocol. To obtain the
170 enzyme extracts, post-diapausing larvae were individually homogenized on ice in 1 ml of a
171 solution of phenyl-methylsulfonyl fluoride (PMSF, 0.4 mM) on phosphate buffer (50 mM, pH
172 7.2). The homogenates were centrifuged for 15 min at 4 ºC and 15 000 ***g***, and the supernatant
173 of each sample was divided into two aliquots that were used as the enzymatic sources of GST
174 and EST (Bouvier et al., 2002).
175
176 *Determination of MFO activity*

177 The MFO activity was determined in black PORVAIR$_{pic}$ 96-well microplates (BIOGEN
178 Científica, Madrid, Spain) using the 7-ethoxycoumarin-*O*-deethylation (ECOD) method

(Bouvier et al., 2002). The microplates were maintained on ice. Each fragment of the post-diapausing larvae was placed individually in a well with 100 µl of sodium phosphate buffer (pH 7.2, 50 mM) and 7-ethoxycoumarin (0.4 mM). As controls, 12 wells were supplied with 100 µl of glycine/ethanol buffer (vol/vol; 0.1 mM, pH 10.4) immediately after the addition of the larval tissue, in order to avoid the reaction. After 4 h of incubation at 30 ºC, the reaction was stopped by adding 100 µl of the glycine/ethanol buffer and the microplate was centrifuged at 2 000 ***g*** for 1 min in order to immerse the larvae fragment and clear the surface of the well for the reading. The 7-hydroxycoumarine fluorescence was quantified with 380 nm excitation and 465 nm emission filters. The results of ECOD activity were expressed in pg of 7-hydroxycoumarine (7OH) per larva per min.

Determination of GST activity

The GST activity was determined in transparent COSTAR 96-well microplates (Corning Life Sciences, Lowell, MA, USA) by UV absorbance using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The microplates were maintained on ice, and each well was supplied with 4 µl of larval extract (equivalent to 0.4 µl insect per well), 185 µl of sodium phosphate buffer (pH 7.2, 50 mM) containing 2 µl of reduced glutathione (GSH) (0.1 M), and 10 µl of CDBN (30 mM). Twelve wells with sodium phosphate buffer only were used as controls. The optical density was measured by absorbance at 340 nm and 30 ºC at time zero (t0), and after 1 min (t1). Results were expressed in mM glutathione conjugated mg per protein per min (Bouvier et al., 2002).

Determination of EST activity

The EST activity was measured on transparent, 96-well GREINER microplates (GREINER Bio-One, Madrid, Spain) by absorbance using β-naphthyl acetate as substrate (Bouvier et al., 2002). Each well was supplied with 90 µl of larval extract (equivalent to 1.8 µl of enzymatic extract per well), and 90 µl of sodium phosphate buffer (pH 6.5, 50 mM) containing β-naphthyl acetate (0.1 mM) (Bouvier et al., 2002). Twelve wells 90 µl of the sodium phosphate buffer only and were used as controls. After 15 min of incubation at 30 ºC, 20 µl of a staining reagent containing 3 g l^{-1} Fast Garnet and 35 g l^{-1} sodium dodecyl sulfate (SDS) were added to the solution. Absorbance of naphthol–Fast Garnet complex was measured after 15 min at room temperature and at 492 nm. The results were expressed in nmol of β-naphthol mg per protein per min.

Determination of the protein content

For the GST and EST analyses, the protein content in each supernatant enzyme extract obtained was measured according to the Bradford method (Bradford, 1976), using bovine serum albumin to build the standard curve.

Data analysis

The insecticide efficacy was calculated by correcting the observed larval mortality with the mortality of the solvent-treated control (Abbott, 1925). The efficacy of each insecticide on each strain and population was compared with that on the susceptible strain S_Spain by a χ^2 test. The MFO, GST, and EST activity was analyzed by ANOVA, followed by a Student-Newman-Keuls (SNK) test to compare means. The relative frequency of resistant individuals (namely RMFO, REST, and RGST) within each population was calculated following the method of Reyes et al. (2007), using the upper activity value of 90% of S_Spain post-diapausing larvae as a threshold. The RMFO, REST, and RGST of each strain and population were compared with that of S_Spain by a χ^2 test. When significant differences were detected, an enzymatic activity ratio was calculated by dividing the value of the enzymatic activity of each population by the value of S_Spain. When no significant differences were detected, the value of the enzymatic activity ratio was 1. When the number of tested populations was greater than nine, the relationship between each insecticide efficacy and each enzymatic activity was analyzed by linear regression analysis, using enzymatic activity as the independent variable. When the number of tested populations was greater than seven, the relationship among the efficacies of each insecticide and among the enzymatic activities was analyzed by correlation analysis.

Results

Insecticide efficacy

Efficacy of the insecticides on post-diapausing larvae of the susceptible strains ranged from 89 to 98% (S_Spain), from 87 to 94% (S_France), and from 78 to 100% (S_Italy) (Table 2). The efficacy of the insecticides on the S_France and S_Italy strains was not significantly different from their efficacy on S_Spain, except in the cases of diflubenzuron on S_Italy (χ^2 = 4.4, d.f. = 1, P = 0.036) and fenoxycarb on S_France (χ^2 = 7.6, d.f. = 1, P = 0.005). In both cases was the efficacy of the insecticides significantly lower than that on S_Spain, but the differences were only about 10% of the efficacy on S_Spain (Table 2).

The efficacy of the insecticides on the population from the organic orchard was not significantly different from their efficacy on S_Spain, except for fenoxycarb (χ^2 = 15.37, d.f. = 1, P<0.001) (Table 2), which was less effective on the organic orchard population than on S_Spain. In the case of the population from the mating disruption + insecticide orchard, only the efficacy of thiacloprid was not significantly lower than its efficacy on S_Spain (χ^2 = 2.9, d.f. = 1, P = 0.08) (Table 2). As for the populations from the conventional orchards, the efficacy of the insecticides was significantly lower than their efficacy on S_Spain, except in the cases of tebufenozide on the Girona population (χ^2 = 0.14, d.f. = 1, P = 0.7), and chlorpyrifos-methyl on the Poal_3 population (χ^2 = 2.41, d.f. = 1, P = 0.12) (Table 2).

Enzymatic activity

Activity of MFOs, GSTs, and ESTs in *C. pomonella* post-diapausing larvae was dependent on the population ($F_{9,190}$ = 7.36, 8.53, and 9.37, respectively, all P<0.0001), but no significant differences were observed among the three susceptible strains and the population from the organic orchard (Table 3). Most of the other field populations (either from the mating disruption + insecticide orchard or from the conventional orchards) showed a significantly higher MFO, GST, and EST activity than that of S_Spain (Table 3). The mating disruption + insecticide orchard population showed MFO and EST activity levels similar to those of the conventional orchard populations mentioned above (Table 3). All the field populations collected from the conventional orchards showed a significantly higher MFO and GST activity than S_Spain, whereas four out of five field populations from conventional orchards showed higher EST activity than S_Spain (Table 3). The values of the enzymatic activity ratio were much higher for MFO activity (ranging from 8.4 to 74.6) than for GST (1.6-2.6) and EST activity (3.3-7.0) (Table 3).

The thresholds used to determine the frequency of resistant individuals in the field populations were 14.14 pg 7OH per larva per min, 13.7 mM of glutathione conjugated mg per protein per min, and 451.39 nmol of β-naphtol mg per protein per min for RMFO, RGST, and REST, respectively. The RMFO, RGST, and REST of S_France, S_Italy, and the organic orchard population were not significantly different from the those of S_Spain, whereas the rest of the field populations showed a higher frequency of resistant individuals than S_Spain, except in two cases (Table 3). For the populations from non-organic orchards, the RMFO ranged from 95 to 100%, the RGST from 20 to 80%, and the REST from 20 to 100%.

Relationships between insecticide efficacy and enzymatic activity

The insecticide efficacy was significantly dependent on MFO activity for all insecticides tested (Table 4, gray area), and especially for azinphos-methyl ($F_{1,8}$ = 14.36, P = 0.005) and thiacloprid ($F_{1,7}$ = 20.74, P = 0.003). It was significantly dependent on the GST and EST activity only in the case of fenoxycarb ($F_{1,8}$ = 8.62, P = 0.022; and $F_{1,8}$ = 7.95, P = 0.027, respectively), and of two organophosphates (azinphos-methyl: $F_{1,8}$ = 7.21, P = 0.027; $F_{1,8}$ = 8.46, P = 0.019; and phosalone: $F_{1,8}$ = 9.36, P = 0.016; $F_{1,8}$ = 5.57, P = 0.045, for EST and GST activities, respectively) (Table 4, gray area).

The efficacies of the insecticides were positively intercorrelated in most cases (Table 4, white area). The efficacy of azinphos-methyl, fenoxycarb, and tebufenozide was significantly correlated with that of the other products tested, except in the case of chlorpyriphos-methyl. On the other hand, the efficacy of phosalone and diflubenzuron showed no significant correlation with that of the neonicotinoid thiacloprid, and the efficacy of chlorpyrifos-ethyl was not correlated with that of chlorpyriphos-methyl and thiacloprid (Table 4, white area). A significant correlation was found between the efficacy of chlorpyriphos-methyl and that of only two other insecticides: diflubenzuron and phosalone. Similarly, the efficacy of thiacloprid was significantly correlated only with that of azinphos-methyl, fenoxycarb, and tebufenozide (Table 4, white area). The EST activity was positively correlated with that of MFO and GST, but the MFO and GST activities were not significantly correlated (Table 4, white area).

Discussion

The susceptibility of the three susceptible strains, S_Spain, S_France, and S_Italy, to the eight insecticides tested may be considered essentially equal, as the efficacy of insecticides on them was significantly different only in two cases out of 16. (Table 2). The efficacies of the different insecticides were very similar to those reported for the susceptible strain S_Switzerland (Pasquier & Charmillot, 2003). Similarly, the MFO, GST, and EST activity of the three susceptible strains was equal (Table 3). The importance of these results lies in the use of a particular susceptible strain to calculate the resistance rate of field populations. As the three tested European susceptible strains are equal, the resistance ratios of field populations from different countries may be compared. Though Silva et al. (2003) found that the geographic origin and the laboratory rearing period could produce differences between susceptible strains, in our case these factors did not influence the results. After these results, S_Spain was selected as the reference susceptible strain for present and further studies.

Only the population from the organic orchard, Boldú, may be considered as a susceptible field population, because the efficacy of all tested insecticides on it was not significantly different from that on S_Spain, with the exception of fenoxycarb. This population showed an enzymatic activity that was not significantly different from that of S_Spain for the three enzymatic systems. In contrast, the population collected from the mating disruption + insecticide orchard (Gimenells, which has a low use of insecticides) showed low susceptibility to all the insecticides tested, except thiacloprid, and higher MFO and EST activities than those of S_Spain, probably because the Gimenells orchard is surrounded by conventional orchards, whereas the Boldú orchard has no other apple or pear fruit orchards in its vicinity. The migration of codling moths among orchards with different management programs has been recorded in several countries (Knight et al., 1994; Fuentes-Contreras et al., 2007), where insecticide resistance has been reported even in populations collected from abandoned orchards due to their proximity to commercial orchards.

All the field populations from conventional orchards were resistant to azinphos-methyl, phosalone, diflubenzuron, and fenoxycarb, with considerable decreases in the insecticide efficacy, which was in most cases lower than 50%. Chlorpyrifos-methyl and chlorpyrifos-ethyl were more toxic to codling moth than phosalone and azinphos-methyl, as has already been reported for European (Reyes et al., 2007; Rodríguez et al., 2010) and North American (Dunley & Welter, 2000) codling moth populations. Azinphos-methyl and phosalone (which have been prohibited in Spain only since 2006 and 2007, respectively) have been much more widely used in Spain than chlorpyrifos-methyl and chlorpyrifos-ethyl. Tebufenozide has been used little in Spain, but it showed low efficacy on field populations. As we found a positive correlation between the efficacy of tebufenozide and the efficacy of six insecticides, including the organophosphate azinphos-methyl and the IGR fenoxycarb, the low efficacy of tebufenozide may be attributed to cross-resistance, as has been reported several times for codling moth (Ioriatti et al., 2007; Knight et al., 2001; Reyes et al., 2007; Sauphanor & Bouvier, 1995) and other lepidopterous pests (Smirle et al., 2002).

The field populations in this study were in general more susceptible to thiacloprid – not yet used in Spain when the populations were collected – than to the other insecticides, although the susceptibility of the conventional orchard populations was slightly lower than that of the susceptible strains. Deciding whether this fact points to an incipient resistance due to cross-resistance needs further investigation with more populations, as we also found a positive correlation among the efficacies of thiacloprid, azinphos-methyl, fenoxycarb, and tebufenozide, and cross-resistance has been reported (Reyes et al., 2007).

Except in one case (EST activity in Poal_4), the field populations showed significantly higher levels of MFO, GST, and EST enzymatic activity than those of the susceptible strain S_Spain, as has been reported in European and American populations (Sauphanor et al., 1997; Bouvier et al., 2002; Ioriatti et al., 2007; Fuentes-Contreras et al., 2007; Reyes et al., 2004, 2007). Involvement of EST activity in the detoxification of insecticides in codling moth has only been observed in Argentinean (Soleño et al., 2003, 2004, 2008) and Spanish field populations (Rodríguez et al., 2010), where higher ratios of EST levels were related to resistance to organophosphates in diapausing larvae of field populations.

In particular, MFOs were involved in the detoxification of the six insecticides for which the regression analyses were carried out, and especially for azinphos-methyl. Esterase and GST activities were only associated with toxicity of fenoxycarb, phosalone, and azinphos-methyl. This is interesting because these insecticides were the ones most widely used in the control programs of *C. pomonella* in the years prior to collection of the diapausing larvae in the study orchard. The association between GST activity and organophosphate resistance has been recorded in European (Reyes et al., 2007; Reyes & Sauphanor, 2008) and Chilean (Fuentes-Contreras et al., 2007) field populations. In an Argentinean field population, however, Soleño et al. (2008) found a poor correlation between GST activity and azinphos-methyl toxicity but an enhanced association between EST activity and azinphos-methyl toxicity. In contrast, in a European field population, Reyes et al. (2007) observed a negative correlation between EST activity in adults and susceptibility to azinphos-methyl in post-diapausing larvae. We suggest that insecticide resistance in our populations of *C. pomonella* may be due to the particular conditions of pest management in the past, supporting the hypothesis that different detoxification mechanisms are selected in different parts of the world in association with resistance to the same insecticides (Reyes et al., 2007). On this point, the Gimenells field population is an interesting example, because it shows two different mechanisms associated with organophosphate resistance: first, a high enzymatic activity of detoxification of MFOs, GSTs, and ESTs (Reyes et al., 2007; Reyes & Sauphanor, 2008; Rodríguez et al., 2010), as in our results; and second, a mutation of acetylcholinesterase (AChE), which reduces susceptibility to organophosphates and carbamates and is only found in Spanish populations (Reyes et al., 2007; Reyes & Sauphanor, 2008). Further research is needed to confirm the presence and frequency of the AChE mutation in Spanish field populations from other areas.

All the conventional orchards from which *C. pomonella* populations were collected showed higher damage at harvest than the economic thresholds (2%), in spite of the

382 insecticides applied. According to our results, these control failures were due to the existence
383 of populations resistant to the main insecticides used.
384
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392
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Table 1 Insecticides and insecticide diagnostic concentrations used to test their efficacy on post-diapausing *Cydia pomonella* larvae

Insecticide	Abbrev.	Purity (%)	Diagnostic concentration (mg l^{-1})[1]	Efficacy on S_Switzerland (%)[1]	Chemical group	Supplier
Azinphos-methyl	Azin-m	93	400	98.3	Organophosphate	Bayer Crop Science, Spain
Chlorpyrifos-ethyl	Chl-e	97	1200	96.7	Organophosphate	Dow AgroScience, Spain
Chlorpyrifos-methyl	Chl-m	97.3	1200	95.6	Organophosphate	Dow AgroScience, Spain
Diflubenzuron	Diflu	90	10000	71.6	Benzoylurea	Afrasa, Spain
Fenoxycarb	Feno	98.5	1	97.8	Fenoxycarb	Syngenta, Spain
Phosalone	Phos	90	3000	99.9	Organophosphate	Rhône-Poulenc Agro SA, Spain
Tebufenozide	Tebu	97	300	94.7	Benzhydrazide	Dow AgroScience, Spain
Thiacloprid	Thia	99.7	500	99.4	Neonicotinoid	Bayer Crop Science, Spain

[1]from Charmillot et al. (2007) for azinphos-methyl, and from Pasquier & Charmillot (2003) for the other insecticides.

Table 2 Efficacy of eight insecticides at their diagnostic concentration (mg l^{-1}) on *Cydia pomonella* post-diapausing larvae from three laboratory strains and seven Spanish field populations

Population	Insecticide[1]							
	Azin-m (400)	Chl-e (1200)	Chl-m (1200)	Diflu (10000)	Feno (1)	Phos (3000)	Tebu (300)	Thia (500)
Susceptible strains								
S_Spain	95	97	97	89	98	95	96	98
S_France	91 ns	94 ns	94 ns	87ns	88**	90 ns	91 ns	94 ns
S_Italy	88 ns	91 ns	100 ns	78*	93 ns	88 ns	90 ns	97 ns
Organic orchard								
Boldú	97 ns	95 ns	100 ns	91ns	81***	89 ns	97 ns	93 ns
Mating disruption + insecticide orchard								
Gimenells	34***	46***			54***	16***	52***	93 ns
Conventional orchard								
Bellvís_2	43***	68***	86**	31***	32***	26***	48***	87**
Girona	33***	81***			58***	58***	97 ns	86**
Poal_1	8***	8***				22***		
Poal_3	34***	66***	92 ns	31***	25***	40***	17***	66***
Poal_4	42***				33***	40***	58***	81***

[1] Mean of four replicates of 10 larvae per insecticide and population.

The efficacy of each insecticide on each population was compared with its efficacy on the susceptible strain S_Spain using χ^2 test (d.f. = 1); ns,

not significant; *P<0.05, **P<0.01, ***P<0.001.

[1]Insecticides: see Table 1 for full names.

Table 3 Mean (± SEM) of MFO, GST, and EST enzymatic activity, Enzymatic Activity Ratio (EAR), and frequency of resistant individuals (RMFO, RGST, and REST) in post-diapausing larvae of *Cydia pomonella* from susceptible strains and Spanish field populations

Populations	MFO (pg 7OH / larva·min)			GST (mM glutathione conj. mg / protein·min)			EST (nmol β-naphthol mg / protein·min)			Frequency of resistant individuals (%)[1]		
	n	Mean activity	EAR[2]	n	Mean activity	EAR[2]	n	Mean activity	EAR[2]	RMFO	RGST	REST
Susceptible strain												
S_Spain	72	7.8 ± 1.0a	1	57	7.6 ± 0.9a	1	46	214.7 ± 29a	1	10	10	10
S_France	40	6.9 ± 0.9a	1	60	7.6 ± 1.2a	1	60	222.3 ± 40a	1	15 ns	0 ns	5 ns
S_Italy	56	8.3 ± 1.3a	1	60	8.1 ± 1.5a	1	60	226.2 ± 50a	1	0 ns	15 ns	5 ns
Organic orchard												
Boldú	20	8.8 ± 1.1a	1	20	7.1a ± 1.2a	1	20	193.8 ± 23.6a	1	10 ns	25 ns	0 ns
Mating disruption + insecticide orchard												
Gimenells	20	187.5 ± 49.0c	24.0	20	8.4 ± 1.2a	1	20	700.6± 111.2b	3.3	95***	20 ns	50**
Conventional orchards												
Bellvís_2	20	186.9 ± 33.4c	23.9	20	20.2 ± 2.0c	2.6	20	1503.3 ± 195.9c	7.0	100***	70***	100***
Girona	20	273.9 ± 65.3c	35.1	20	16.5 ± 2.6bc	2.2	20	830.9 ± 98.0b	3.9	100***	45**	80***
Poal_1	20	468.3 ± 81.4d	60.0	20	15.3 ± 1.4bc	2.0	20	766.9 ± 84.5b	3.6	100***	80***	90***
Poal_3	20	582.1 ± 88.0d	74.6	20	13.0 ± 1.9b	1.7	20	978.2 ± 103.0b	4.6	100***	45**	90***
Poal_4	20	65.2 ± 5.9b	8.4	20	12.4 ± 1.5b	1.6	20	324.7 ± 33.3a	1	100***	50**	20 ns

n = number of replicates.

Means followed by the same letter in the same column are not significantly different (Student-Newman-Keuls test: $P<0.05$).

Frequency of resistant individuals of each population was compared with that of S_Spain using a χ^2 test (d.f. = 1); ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

[1] Thresholds: 14.14 pg 7OH per larva per min, 13.7 mM of glutathione conjugated mg per protein per min, and 451.39 nmol of β-naphtol mg per protein per min, for RMFO, RGST, and REST, respectively.

[2]EAR = enzymatic activity of the field population divided by the enzymatic activity of the susceptible strain S_Spain, when the enzymatic activities were significantly different.

Table 4 Matrix of correlation coefficients (white area) and determination coefficients (gray area) of the correlation analyses and of the linear regression analyses of insecticide efficacy on enzymatic activity for three susceptible strains and seven field populations of *Cydia pomonella*

	Azin-m	Chl-e	Chl-m	Diflu	Feno	Phos	Tebu	Thia	MFO	GST	EST
Azin-m	1	0.86**	0.80ns	0.99***	0.88**	0.92***	0.70*	0.68*	0.64**	0.51*	0.47***
Chl-e		1	0.80ns	1.00***	0.77*	0.85**	0.77*	0.44ns	0.49*	0.2ns	0.23ns
Chl-m			1	0.81*	0.87ns	0.87*	0.70ns	0.50ns			
Diflu				1	0.96**	0.98***	0.95**	0.80ns			
Feno					1	0.87**	0.85**	0.83**	0.52*	0.52*	0.55*
Phos						1	0.79*	0.53ns	0.43*	0.41*	0.54*
Tebu							1	0.79*	0.57*	0.18ns	0.41ns
Thia								1	0.75**	0.25ns	0.26ns
MFO									1	0.56ns	0.65*
GST										1	0.87**
EST											1

*P<0.05; **P<0.01; ***P<0.001; ns = not significant.

Insecticides: see Table 1 for full names. Enzymes: MFO, mixed-function oxidases; GST, glutathione transferases; and EST, esterases.